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Thanks,

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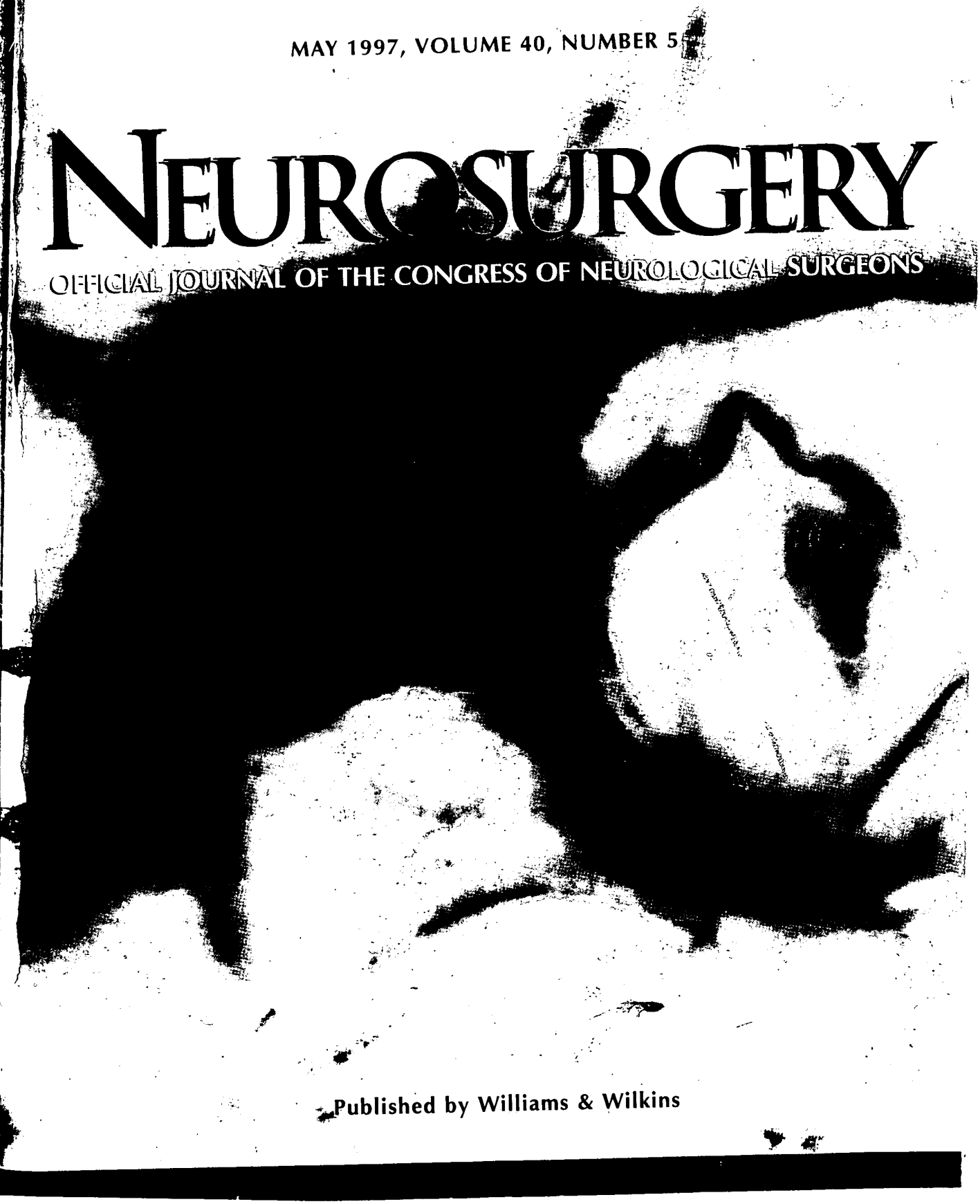

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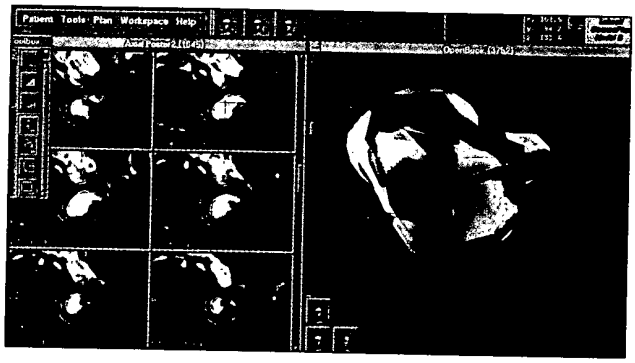

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Meningiomas: Role of Vascular Endothelial Growth Factor/Vascular Permeability Factor in Angiogenesis and Peritumoral Edema

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OBJECTIVE: Vascular endothelial growth factor (VEGF)/vascular permeability factor (VPF) is a potent angiogenic growth factor implicated in the tumor angiogenesis/metastasis of a number of human cancers. Activation of receptors for VEGF/VPF is specifically mitogenic to endothelial cells and increases their permeability. Although extensive literature exists regarding VEGF/VPF in human astrocytomas, little is known about its potential biological role(s) in meningiomas. Our interest in meningiomas was initiated by the observation that some meningiomas are extremely vascular and are occasionally associated with a considerable degree of peritumoral brain edema, both potentially related to the biological attributes of VEGF/VPF.

METHODS: As a first test of this hypothesis, we examined a cohort of 18 meningiomas for expression of VEGF/VPF at the messenger ribonucleic acid and protein levels and correlated expression with pathological characteristics, vascularity, and degree of peritumoral edema.

RESULTS: The majority of meningiomas expressed VEGF/VPF at both the messenger ribonucleic acid and protein levels. Corresponding serial sections were stained with an endothelial cell marker to obtain a microvascular density count, which positively correlated ($P = 0.0005$) with expression of VEGF/VPF. Furthermore, meningiomas with a large amount of peritumoral edema, as determined from the preoperative computed tomographic scans or magnetic resonance imaging scans, had elevated expression of VEGF/VPF ($P = 0.05$).

CONCLUSION: These data suggest that VEGF/VPF may play a role in both meningioma vascularity and peritumoral edema. (Neurosurgery 40:1016–1026, 1997)

Key words: Angiogenesis, Meningioma, Peritumoral edema, Reactive astrocytes, Vascular endothelial growth factor, Vascular permeability factor

Meningiomas overall are of variable vascularity, ranging from relatively avascular to highly vascular angiomatous meningiomas. Regional heterogeneity in the degree of vascularity exists even within individual meningiomas, although histological evidence of florid neoangiogenesis is much less common than with malignant astrocytomas. Another clinically relevant feature of some meningiomas is the disproportionately large amount of peritumoral brain edema, the mechanisms of which are not fully explained by size, histological subtype, location, or proximity to venous channels (1, 30, 43). Both of these observations, i.e., tumor vascularity and tumor-associated edema, are attributes linked to the functions of vascular endothelial growth factor (VEGF)/vascular permeability factor (VPF). Our interest in determining the relationship between VEGF/VPF and these two properties of meningiomas was kindled by the care of Patient 1. This elderly diabetic patient, with a history of significant coronary insufficiency, presented with confusion and headache. Contrast-enhanced computed tomographic (CT) scans revealed two anterior fossa meningiomas; the larger left convexity tumor was asymptomatic (*Fig. 1B*), whereas the smaller right basal tumor was the lesion responsible for the symptoms because of an extensive amount of peritumoral edema (*Fig. 1A*). Steroid therapy for the patient was begun,

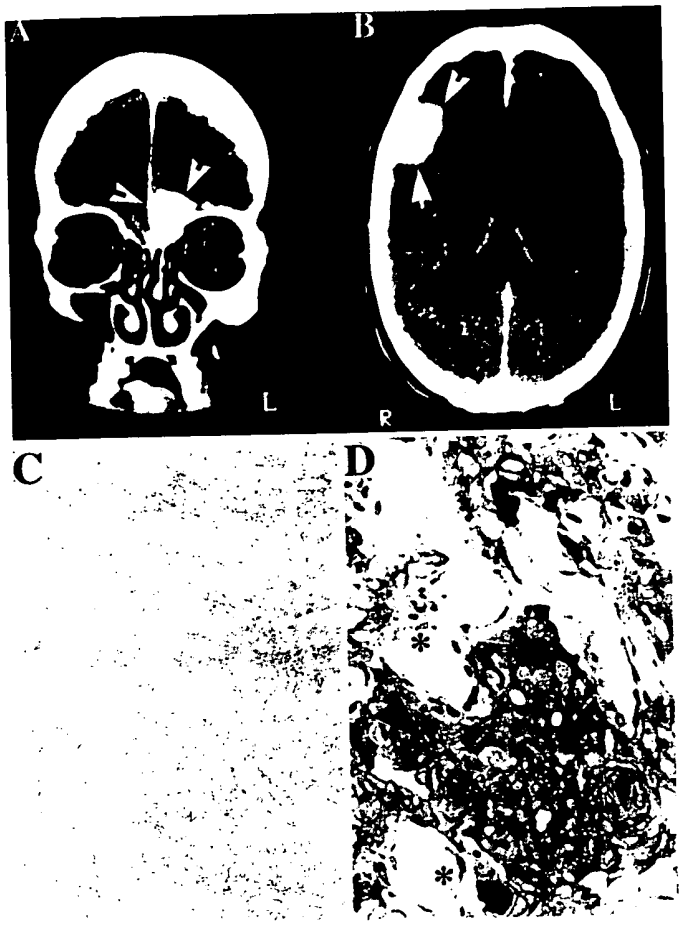

FIGURE 1. ***A*** **and** ***B*****, coronal and axial contrast-enhanced CT scans of Patient 1 (the index case). The symptomatic meningioma with prominent peritumoral edema (*A, arrowheads*) was resected, whereas the contralateral larger but asymptomatic meningioma with little peritumoral edema was obtained at autopsy (*B, arrowheads*). *C*, VEGF/VPF immunohistochemistry (original magnification, ×150) of the asymptomatic meningioma (*), demonstrating low levels of positivity in the tumor. *D*, VEGF/VPF immunohistochemistry (original magnification, ×250) of the symptomatic meningioma, with high levels of positivity in the meningothelial cells (*arrowheads*) and not in the tumor endothelial cells (*asterisks*).**

without resolution of his clinical symptoms or radiological resolution of the peritumoral edema after 2 weeks but with significant side effects related to his uncontrolled diabetes. The patient proceeded to operative resection of the right basal meningioma, with an uneventful intraoperative course, but, unfortunately, he suffered a fatal myocardial infarction in the intensive care unit. We obtained flash-frozen samples of the symptomatic right basal meningioma intraoperatively, whereas the asymptomatic left convexity meningioma (*Fig. 1*) and peritumoral brain tissue from both meningiomas were obtained at autopsy.

VEGF was initially isolated from bovine pituitary follicular stellate cells and was given its name because of its potent and specific mitogenic stimulation of endothelial cells (14, 19, 33). VPF was independently isolated and characterized, as a potent inducer of vascular permeability, from tumor ascites fluid and conditioned media of numerous tumor cell lines (48, 49). It was subsequently established that VEGF and VPF were identical M_r 45,000 glycoproteins, belonging to the platelet-derived growth factor (PDGF) family, with which they share approximately 20% homology (56). Four isoforms of VEGF/VPF, resulting from alternate splicing, have now been identified, with the two smallest isoforms ($VEGF_{121}$ and $VEGF_{165}$) being highly secreted because of the presence of a signal peptide and $VEGF/VPF_{165}$ being the predominant isoform expressed (29). The biological functions of VEGF/VPF are mediated through two high-affinity protein receptor tyrosine kinases identified initially in mice, Flt-1 and Flk-1 (the human counterpart of the latter is known as KDR), of which Flk-1/KDR seems to be more important biologically (4, 16, 55). These two receptors are primarily expressed on vascular endothelial cells, accounting for the main biological function of VEGF/VPF, as a highly potent angiogenic growth factor in normal development and various benign and neoplastic pathological states (9, 11, 36). The expression of VEGF/VPF and that of its receptors are coordinately up-regulated to promote angiogenesis in the developing central nervous system (CNS) (5, 9, 36), with low to undetectable levels of both the ligand and the receptors in the normal adult CNS (19). The importance of VEGF/VPF in development has been recently underscored by the embryologically lethal vascular abnormalities observed in knockout mice lacking Flt-1 and Flk-1 (24, 50). In addition to its pivotal role in angiogenesis, VEGF/VPF is a potent inducer of microvascular permeability, which is integral to the angiogenic process, having 10,000 to 50,000 times the activity of histamine in Miles bioassays (13, 17).

The progression of a low-grade astrocytoma to a highly vascularized glioblastoma multiforme (GBM) is associated with increased VEGF/VPF messenger ribonucleic acid (mRNA) expression by the astrocytoma cells, especially those surrounding the perinecrotic zones (40, 51). The florid endothelial proliferation characteristic of malignant astrocytomas has been linked to up-regulation of KDR mRNA by in situ hybridization (a good antibody to KDR is not yet available), with VEGF/VPF protein (not mRNA) co-localizing to the endothelial cells expressing KDR (18, 19, 35, 40). These findings are consistent with a paracrine role for VEGF/VPF in astrocytoma angiogenesis, with the highly secreted VEGF/VPF being made by the astrocytoma cells and stimulating the proliferation of the tumor-associated vascular endothelial cells. This hypothesis was further supported by the demonstration that neutralizing monoclonal antibodies, antisense constructs against VEGF/VPF, and dominant negative mutants against

Flk-1/KDR are capable of decreasing astrocytoma growth, both in vitro and in vivo, by interrupting the VEGF/VPF paracrine loop (3, 31, 35, 52). Although the role of VEGF/VPF has been examined in astrocytic tumors and some other glial tumors (2, 15, 54, 58), to date, there is scant information on VEGF/VPF expression in meningiomas (27, 44), which are the second most common primary CNS tumors among adults.

MATERIALS AND METHODS

Pathological specimens

Eighteen meningioma specimens, which showed varying degrees of preoperative peritumoral edema (as evaluated in the presenting CT scans and magnetic resonance imaging [MRI] scans, before initiation of steroid therapy) and for which flash-frozen specimens were available for analysis from the University of Toronto Nervous System Tumor Bank (*Table 1*), were chosen. All of the 18 meningioma specimens were fixed in 4% formalin and embedded in paraffin, and 8-μm sections were cut for immunohistochemical analysis; the flash-frozen sections were used for Northern and in situ hybridization analysis.

Immunohistochemistry and tumor vascularity

All paraffin-embedded tumor sections underwent routine staining with hematoxylin and eosin. VEGF/VPF immunohistochemistry was performed with a rabbit polyclonal antiserum (18), at a dilution of 1:50, by using a peroxidase/antiperoxidase detection system (Vectastain ABC kit; Burlingame, CA), with no pretreatment or antigen retrieval. A semiquantitative grade from 0 through 4 was assigned in a blinded fashion, with negative control sections not receiving primary antibody (Grade 0) and paraffin-embedded GBM sections serving as positive controls (Grade 4). Factor VIII (polyclonal antibody used at 1:100 dilution; DAKO, Denmark) staining was used as a marker of endothelial cells, to assess tumor vascularity. Antiserum to glial fibrillary acidic protein (bovine polyclonal antibody used at 1:100 dilution; DAKO) was used to stain the reactive astrocytes, which were also stained in parallel sections with the antibody to VEGF/VPF, in the peritumoral brain (*Fig. 2*).

Meningioma vascularity was assessed by using both a semiquantitative grading system and a more formal, microvascular density grading scheme. The degree of tumor vascularity, as detected on the hematoxylin/eosin-stained sections, was evaluated in a blinded fashion and graded from 0 to 4 (with Grade 4 indicating the most vascular tumors) (*Table 1*). In addition, sections stained with Factor VIII were evaluated under high-power magnification, and a quantitative microvascular density count (*Table 1*) was obtained by averaging the number of tumor vessels in four high-power fields.

VEGF/VPF mRNA analysis

Northern analysis

Flash-frozen tumor specimens were homogenized in guanidinium isothiocyanate buffer and processed through a cesium chloride gradient to extract total ribonucleic acid (RNA). Ten micrograms of total RNA were separated on a 1% dena-

TABLE 1. Meningiomas: Clinical, Pathological, Radiological, Vascularity, and Vascular Endothelial Growth Factor/Vascular Permeability Factor Expression Data[a]

Sample	Age	Gender	Pathology	Maximal Peritumoral Edema (cm)	Maximal Tumor Diameter (cm)	Meningioma Vascularity		VEGF/VPF	
						Vascularity Grade (0–4)	Microvascular Density (vessels/×400 field)	Immunohistochemistry (0–4)	mRNA (0–4)
WM	62	M	Transitional	0.5	2.5	1	7	1	1
JL	80	M	Transitional	10.0	5.0	NA	NA	NA	1
DS	83	F	Transitional	5.0	5.0	NA	NA	NA	1
LH	82	M	Transitional	3.5	1.5	NA	NA	4	2
GR	58	M	Transitional	0.2	2.5	2	12	2	1
IK	62	M	Transitional	NA	NA	3	15	4	2
JC	83	M	Transitional	6.0	3.0	4	30	3	4
JC[b]	83	M	Transitional	0	5.0	2	5	1	NA
DD	54	M	Transitional	NA	NA	2	7	2	2
MP	56	F	Transitional	0.1	0.9	3	20	3	4
ET	61	F	Transitional	1.0	1.5	NA	NA	0	0
RK	75	F	Transitional	NA	NA	NA	NA	2	3
LS	75	F	Syncytial	0.5	7.0	NA	NA	4	3
LP	44	F	Syncytial	NA	NA	4	25	3	4
ER	77	F	Transitional	2.5	2.5	2	6	0	0
KN	76	M	Transitional	3.5	1.5	1	5	2	2
RS	47	F	Transitional	NA	NA	0	3	1	0
SE	47	F	Fibroblastic	NA	NA	NA	NA	NA	0

[a] **VEGF, vascular endothelial growth factor; VPF, vascular permeability factor; NA, data not available.**
[b] **Autopsy specimen.**

FIGURE 2. High-power image (original magnification, ×600) of a reactive astrocyte located in the peritumoral edematous brain tissue obtained at autopsy from the side of the symptomatic anterior cranial fossa floor meningioma (Patient 1). There is strong VEGF/VPF immunopositivity in the cell body and processes (*arrows*) of these glial fibrillary acidic protein-positive (data not shown) reactive astrocytes.

turing formaldehyde-agarose gel and transferred to BioTrans nylon membranes (ICN, CA). The membrane was hybridized with a randomly primed, ^{32}P-labeled, human VEGF complementary deoxyribonucleic acid probe (Multiprime; Amersham, Arlington Heights, IL), which had been obtained by reverse transcription-polymerase chain reaction from the human U373 glioblastoma cell line (American Type Culture Collection, Rockville, MD). Oligonucleotides used to amplify the coding region and a portion of the 3'-untranslated region were as follows: Primer 1, 5'-ATGAACTTTCTGCTGT-3'; Primer 2, 5'-GTCGATGGTGATGGTGTGGTG-3'. The polymerase chain reaction product was cloned into pGEM-T (Promega) and sequenced, to confirm the lack of mutations. After overnight hybridization at 55°C, the blots were washed three times with 0.1× standard saline citrate (SSC)/1% sodium dodecyl sulfate at 65°C and were exposed to Kodak X-OMAT x-ray film for 1 week. The VEGF/VPF mRNA transcript was detected at 4.2 kilobases (*Fig. 3*), and the loading was verified with a 36B4 human ribosome-associated complementary deoxyribonucleic acid probe (34). The autoradiograms were quantified by using densitometry, and the VEGF/VPF mRNA signal was normalized to the signal of the housekeeping gene 36B4, to account for any mRNA loading differences among the tumors. Tumors with normalized VEGF/VPF mRNA signals between 75 and 100% of the highest value were denoted as Grade 4, with the rest of the grading scheme for VEGF/VPF mRNA in *Table 1* being as follows: Grade 3, 50 to 75%; Grade 2, 25 to 50%; Grade 1, 10 to 25%; Grade 0, <10%.

In situ hybridization

In situ hybridization was performed on two selected specimens, with high (Patient 1) and moderate (Patient 2) VEGF/

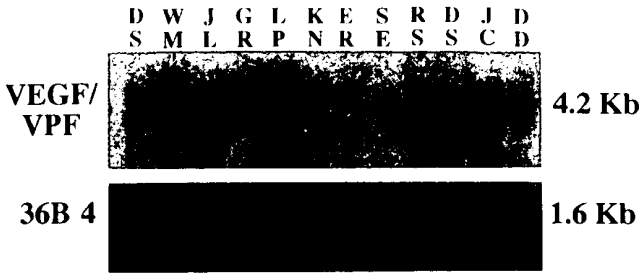

FIGURE 3. Northern analysis for VEGF/VPF mRNA (4.2 kilobases) in flash-frozen surgical meningioma specimens. Twelve lanes representing 11 different tumor specimens are shown, with the index case (Patient 1) noted as *JC* (Patient 2 is noted as *GR*). Total RNA loading was controlled for by hybridization to the housekeeping gene 36B4 (1.6 kilobases), which codes for a ribosome-associated cytoplasmic protein. The amount of VEGF/VPF mRNA expression quantified by densitometry was normalized to the 36B4 signal and graded (*Table 1*). VEGF/VPF mRNA was expressed by the majority of meningioma specimens but to varying extents, with particularly high levels in the index case.

VPF mRNA expression (based on the Northern analysis data) (*Table 1* and *Fig. 3*). Cryosections from the tumor bank with both antisense and sense control riboprobes for VEGF/VPF were used, with the previously described protocol (24a). In brief, the 6-μm cryosections were hybridized overnight at 50°C with ^{35}S-labeled riboprobes in the following mixture: 0.3 mol/L NaCl, 0.01 mol/L Tris, pH 7.6, 5 mmol/L ethylenediaminetetraacetic acid, 0.02% (w/v) Ficoll, 0.02% (w/v) polyvinylpyrrolidone, 0.02% (w/v) bovine serum albumin (Fraction V), 50% formamide, 10% dextran sulfate, 0.1 mg/ml yeast transfer RNA, and 0.01 mol/L dithiothreitol. Posthybridization washes included 2× SSC, 50% formamide, 10 mmol/L dithiothreitol at 50°C, 4× SSC, 10 mmol/L Tris, 1 mmol/L ethylenediaminetetraacetic acid with 20 μg/ml ribonuclease at 37°C, 2× SSC, 50% formamide, 10 mmol/L dithiothreitol at 65°C, and 2× SSC. Slides were then dehydrated through graded alcohols containing 0.3 mol/L ammonium acetate, dried, coated with Kodak NTB 2 emulsion, and stored in the dark at 4°C for 2 weeks. The emulsion was developed with Kodak D19 developer, and the slides were counterstained with hematoxylin (*Fig. 4*). Details of the selection and preparation of the antisense, single-stranded, ^{35}S-labeled, VEGF/VPF RNA probe and its sense control probe were previously described (12). The antisense probe hybridizes specifically with a region of VEGF/VPF mRNA common to all four VEGF/VPF splicing variants.

Evaluation of preoperative peritumoral edema

For 11 of 17 meningioma cases pathologically examined, initial preoperative CT and/or MRI scans, obtained before institution of steroid therapy, were available (*Table 1*). Contrast-enhanced CT scans were available for only 9 of 11 patients, with the largest diameter of the hypointense signal on the plane (coronal or axial) from which the tumor diameter was

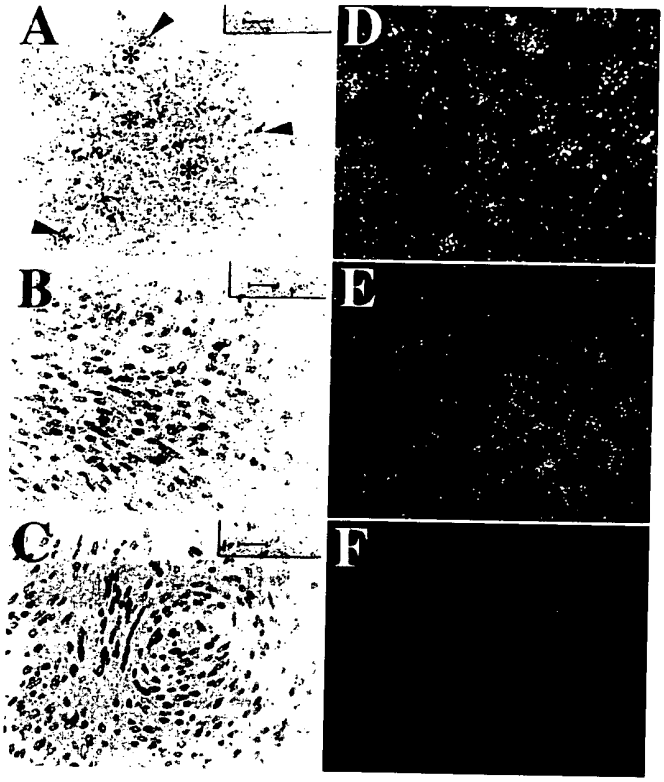

FIGURE 4. Light-field (*A*, *B*, and *C*) and dark-field (*D*, *E*, and *F*) images of in situ hybridization (original magnification, ×800) for VEGF/VPF mRNA, in snap-frozen surgical meningioma specimens (*A* and *D*, Patient 1, antisense probe; *B* and *E*, Patient 2, antisense probe; *C* and *F*, Patient 2, sense probe negative control). The specimen from Patient 1 demonstrates high levels of VEGF/VPF mRNA expression by the meningothelial tumor cells (*arrowheads*) surrounding the numerous vascular channels (*asterisks*), reflecting the high microvascular density count of 30 in this tumor. Meningothelial tumor cells from Patient 2 expressed moderate amounts of VEGF/VPF mRNA, corresponding to a microvascular density of 12.

obtained being recorded as the amount of peritumoral edema corresponding to that meningioma. For the two patients for whom contrast-enhanced MRI scans were available, the amount of associated peritumoral edema for each meningioma was assessed by determining the largest diameter of the high T2-weighted MRI signal (axial or coronal) corresponding to the plane from which the tumor diameter measurement was derived. In 10 tumors, the peritumoral edema:tumor diameter ratio could be derived and compared with VEGF/VPF mRNA expression (*Fig. 5B*).

Statistical analysis

Fisher's *r* to *z* transformation correlation analysis (with Statview software and a Macintosh PowerMac) was used to analyze the relationship between meningioma microvascular density count and edema/tumor ratio versus VEGF/VPF mRNA expression.

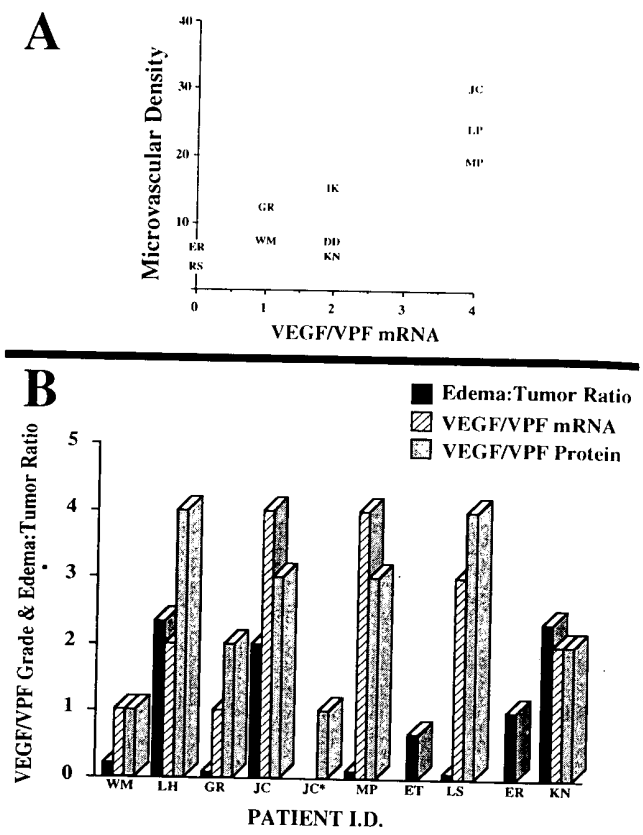

FIGURE 5. *A*, relationship between microvascular density and VEGF/VPF mRNA expression, as tabulated in *Table 1*. Tumors that were more vascular, with a higher microvascular density, expressed increased amounts of VEGF/VPF mRNA (positive correlation at $P = 0.0005$ by Fisher's *r* to *z* correlation analysis). *B*, relationship between VEGF/VPF expression and degree of peritumoral edema, as measured by taking the ratio of maximal edema:maximal tumor diameter from *Table 1*. All three cases with prominent peritumoral edema (edema:tumor ratio > 2) had high levels of VEGF/VPF mRNA and protein expression, with five of the tumors having little tumor edema (ratio < 2) expressing small amounts of VEGF/VPF. The direct correlation between edema and VEGF/VPF expression was not observed in all meningiomas; in two cases (*MP* and *LS*), high levels of expression of VEGF/VPF were associated with very little peritumoral edema. This suggests that although VEGF/VPF expression is required for peritumoral edema, other mechanical factors, as discussed in the text, modify this biological effect.

RESULTS

The 18 supratentorial meningiomas were from eight men and nine women, ranging from 44 to 83 years of age (*Table 1*). There were 14 transitional meningiomas, two syncytial meningiomas, and one fibroblastic meningioma. The degree of vascularity, peritumoral edema, or VEGF/VPF expression did not vary with the histological subclassification of the meningiomas (*Table 1*). By Northern analysis, variable levels of

VEGF/VPF mRNA were detected in 13 of 17 flash-frozen meningioma specimens (*Fig. 3*). The VEGF/VPF signals in the autoradiograms were quantitated by densitometry, normalized for loading (signal from the housekeeping gene 36B4), and graded on a scale from 0 through 4, as described under Materials and Methods. The degree of VEGF/VPF mRNA expression in the meningiomas was positively correlated with VEGF/VPF protein expression, as determined semiquantitatively by immunohistochemistry, for the majority of tumors (*Table 1*). To confirm that the VEGF/VPF mRNA was localized to the meningiothelial tumor cells, in situ hybridization (*Fig. 4*) was undertaken with two representative, flash-frozen tumor samples (Patient 1, index patient; Patient 2, patient with moderate VEGF/VPF expression). VEGF/VPF protein expression, as detected by immunohistochemistry, was positive in the 12 of 14 meningiomas that could be assessed (*Table 1*). In those 12 cases, VEGF/VPF was expressed by the meningothelial tumor cells; however, the degree of expression varied among the tumors. Immunopositivity was specific to the tumor cells and was not present in tumor stroma, connective tissue, or endothelial cells (*Figs. 6* and *7*). The expression of VEGF/VPF was generally diffuse throughout the meningiomas and did not differ among histological subtypes. However, in several meningiomas, an additional increase in expression of VEGF/VPF was localized in the perivascular tumor meningothelial cells (*Fig. 7C*).

Tumor vascularity was graded by assigning a semiquantitative vascularity grade (Grades 0–4) in a blinded fashion and by determining a quantitative microvascular density count (*Table 1*). Meningioma vascularity could be assessed by using these two parameters in 10 of 17 meningiomas and in the asymptomatic contralateral meningioma specimen from our index patient. The correlation between microvascular density count and VEGF/VPF mRNA expression in the meningiomas, by Fisher's r to z transformation analysis, was 0.867, which was significant at $P = 0.0005$. This correlation was best illustrated between the two meningioma samples from our index patient. In the symptomatic tumor (Patient 1), the microvascular density count was 30 vessels/×400 field, with a VEGF/VPF mRNA expression score of 4 (*Table 1*). In addition, immunohistochemistry with antiserum to Factor VIII demonstrated, in this meningioma, occasional foci of hyperprolifera-

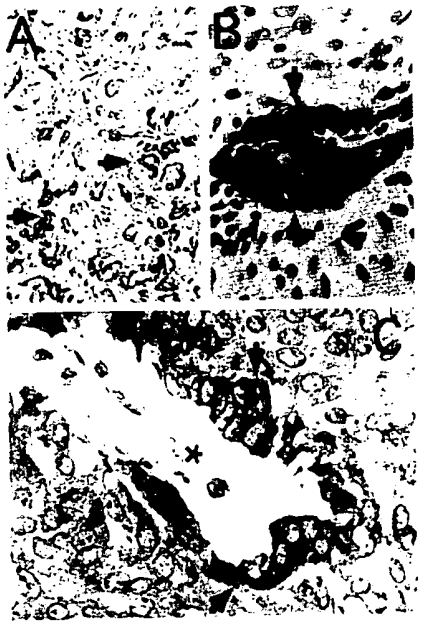

FIGURE 7. Highly vascularized meningioma (Patient 1) stained with Factor VIII (*A*, original magnification, ×200; *B*, original magnification, ×600) and VEGF/VPF (*C*, original magnification, ×600). Vascular endothelial cells stain positively with Factor VIII, with focal areas of endothelial hyperproliferation and some glomeruloid formations (*A* and *B*, *arrows*). These vascularized meningiomas with prominent and active angiogenesis expressed high levels of VEGF/VPF, which were often increased in the perivascular meningothelial cells (*C*, *arrows*). Note the absence of VEGF/VPF immunoreactivity in the blood vessel stroma and endothelial cells (*C*, *asterisk*). This is consistent with a paracrine action of VEGF/VPF, produced by meningothelial cells, acting on vascular endothelial cells.

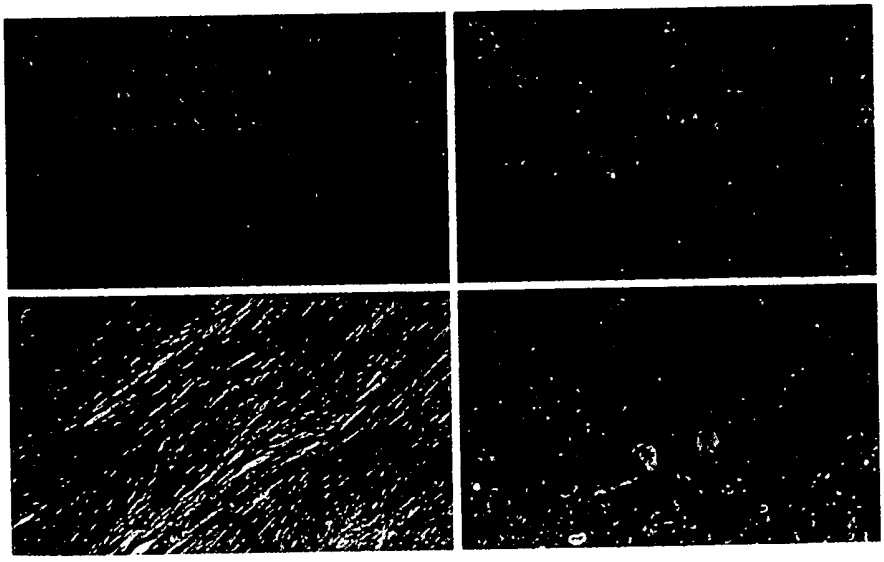

FIGURE 6. Meningiomas showing the range of vascularity and corresponding levels of VEGF/VPF protein by immunohistochemistry. *A* and *B*, hematoxylin and eosin; *C* and *D*, polyclonal antibody that recognizes all isoforms of VEGF/VPF. *A* and *C*, meningioma with low vascularity and minimal VEGF/VPF immunoreactivity (original magnification, ×100). *B* and *D*, meningioma with high vascularity with correspondingly higher levels of immunoreactive VEGF/VPF (original magnification, ×150). *, endothelial cells in vascular channels (VEGF/VPF-negative); *arrowheads*, VEGF/VPF-positive meningothelial tumor cells.

tive endothelial cells (*Fig. 7B*), which are rarely observed in meningiomas and are indicative of active angiogenesis. In contrast, the contralateral asymptomatic tumor (*Fig. 7*), without peritumoral edema, had a microvascular density count of 5 vessels/×400 field, with minimal expression (Grade 1) of VEGF/VPF protein (Table 1), as determined by immunohistochemistry (mRNA levels could not be determined because this was an autopsy specimen).

In addition to tumor vascularity, the degree of peritumoral edema, as indicated in CT or MRI scans before institution of steroids (*Table 1*), was proportional to VEGF/VPF mRNA/protein expression in some but not all of the 10 meningiomas for which this could be assessed (*Fig. 5B*). This was best illustrated by comparing the increased VEGF/VPF expression in the symptomatic tumor from our index case (Patient 1, peritumoral edema/maximal tumor diameter = 2) and the asymptomatic contralateral meningioma (peritumoral edema/maximal tumor diameter = 0) with minimal VEGF/VPF expression. Two other meningiomas for which the peritumoral edema was at least twice the maximal tumor diameter (*Fig. 5, LH* and *KN*) also expressed abundant levels of VEGF/VPF mRNA and protein. In addition, several tumors with minimal to absent peritumoral edema (*Fig. 5, WM, ET, ER,* and *GR*) had low levels of VEGF/VPF mRNA expression. When these eight specimens were analyzed separately, the correlation between edema:tumor ratio and VEGF/VPF mRNA expression levels was 0.700 (significant at $P = 0.05$). However, in two meningiomas (*Fig. 5, MP* and *LS*), abundant VEGF/VPF expression with accompanied tumor vascularity was present, without causing significant peritumoral edema. When all 10 meningioma specimens were analyzed together, the correlation was not significant. Other parameters, such as tumor location, size, and histological subtype of the meningiomas, did not correlate with the amount of peritumoral edema (data not shown).

Because high-grade astrocytoma cells are known to express VEGF/VPF (58), we postulated that reactive astrocytes present secondary to the peritumoral edema may also be positive for VEGF/VPF. The peritumoral edematous brain tissue surrounding the symptomatic meningioma (Patient 1) and corresponding nonedematous brain tissue from the contralateral asymptomatic meningioma, both obtained at autopsy, were examined by immunohistochemistry for glial fibrillary acidic protein and VEGF/VPF expression (*Fig. 2*). The peritumoral edematous brain tissue showed strong immunohistochemical positivity for VEGF/VPF within the reactive astrocytes (*Fig. 2*). In adjacent sections, the reactive astrocytes were strongly positive for glial fibrillary acidic protein (data not shown). The nonedematous brain tissue surrounding the larger asymptomatic contralateral meningioma had a much smaller number of reactive astrocytes and was essentially similar to normal brain tissue, with minimal VEGF/VPF reactivity. VEGF/VPF expression by reactive astrocytes was not restricted to peritumoral brain, because it was observed in a variety of other pathological conditions (ongoing work), including those associated with cerebral infarction.

DISCUSSION

Meningiomas are largely benign tumors; however, as with all solid neoplasms having more than approximately 10^6 cells, neoangiogenesis involving production of angiogenic factors and/or decreased production of angiogenesis inhibitors is crucial for further tumor growth (8, 23, 28). This study demonstrates expression of VEGF/VPF, a growth factor with specific and potent endothelial mitogenic (and, hence, angiogenic) activity, by the majority of meningiomas. Meningioma size, location, and histological subtype and patient characteristics did not correlate with the degree of VEGF/VPF expression. However, the degree of meningioma vascularity positively correlated with expression of both VEGF/VPF mRNA and protein, suggesting that VEGF/VPF was involved in meningioma neoangiogenesis (*Fig. 5A*). A similar correlation between VEGF/VPF expression and meningioma vascularity was reported in one previous study (44). VEGF/VPF was expressed diffusely by the tumorigenic meningothelial cells and not by the endothelial cells or stromal elements, although there was increased expression in the perivascular meningothelial cells sometimes associated with small areas of hyperproliferative endothelial cells or small glomeruloid formations (*Fig. 7C*), similar to the pattern demonstrated in human GBMs. This suggests ongoing dynamic angiogenesis, as is observed in GBMs, consistent with an angiogenic effect of VEGF/VPF acting in a paracrine fashion (i.e., produced by the tumorigenic meningothelial cells and stimulating vascular endothelial cells).

Additional proof of a paracrine mechanism of VEGF/VPF action would require demonstration that the VEGF/VPF receptors (Flt-1 and Flk-1/KDR) are up-regulated on meningioma vascular endothelial cells, compared with normal brain vasculature, in which they are not detectable. Antibodies to VEGF/VPF receptors that are suitable for immunohistochemistry are currently not available, necessitating analysis at the mRNA level on frozen specimens. Reprobing of the VEGF/VPF Northern blots (*Fig. 3*) with the receptor probes (data not shown) suggested that Flk-1/KDR (the most biologically relevant VEGF/VPF receptor) is up-regulated in meningiomas, consistent with a paracrine mechanism. In addition, a previous report documented expression of both Flk-1/KDR and Flt-1 by meningioma vascular endothelial cells, with in situ hybridization experiments (2). We acknowledge that our results demonstrating expression of VEGF/VPF by the meningothelial cells in proportion to tumor vascularity and the presence of cognate receptors in the endothelial cells suggest but do not conclusively prove a VEGF/VPF-mediated paracrine cause of meningioma vascularization. Experiments blocking the VEGF/VPF paracrine stimulatory loop with antibodies, inhibitory mutants, or pharmacological agents directed against the ligand or receptors, similar to the experiments undertaken with astrocytomas, will be required to further test this hypothesis (35).

VEGF/VPF exists in four isoforms, because of alternative exon splicing of the mRNA, with the smallest two isoforms ($VEGF/VPF_{121}$ and $VEGF/VPF_{165}$) being highly secreted (29). Our study did not differentiate between the four isoforms; however, previous studies showed that the majority of tumors

contain all isoforms, with the VEGF/VPF_{165} subtype being the most prevalent (19). The factors regulating VEGF/VPF gene expression in tumor systems are under study. Hypoxia is a strong transcriptional stimulant, as are other mitogenic growth factors, such as PDGF and epidermal growth factor (EGF) (21, 25, 37, 51, 57). Overexpression of EGF and PDGF and their respective receptors has been documented in meningiomas and postulated to contribute to their growth (6, 53). This raises the possibility that these growth-promoting factors not only act as mitogens for the tumorigenic meningothelial cells but also help recruit tumor vessels by up-regulating VEGF/VPF, with both components being vital for the overall growth of the meningioma. A similar molecular pathogenic mechanism can be postulated for astrocytoma growth, with relevant mitogens stimulating uncontrolled proliferation of the astrocytoma cells and, indirectly, the endothelial cells, by VEGF/VPF. Hypoxia leading to tumor necrosis, a pathological feature that is occasionally observed in atypical, extremely large, or malignant meningiomas, would be another important VEGF/VPF transcriptional stimulant in astrocytomas.

Recently, the transcriptional up-regulation of VEGF/VPF has been shown to be mediated by activation of Ras, a major intracellular signal transduction protein (42). The majority of the mitogenic signals (or perhaps the differentiation signals, depending on cell type and environment) resulting from activation of PDGF, EGF, and a variety of other cell surface receptors require Ras activation. This has been demonstrated by experiments in which levels of activated Ras are measured after growth factor stimulation or in which Ras activity is blocked (32, 38, 39, 41, 45–47). The importance of Ras activation is further demonstrated; oncogenic mutations of Ras, in which Ras is constitutively activated, are observed in approximately 30% of all human neoplasms, although they do not occur in astrocytomas or meningiomas (7). We have demonstrated that although astrocytomas do not harbor oncogenic Ras mutations, levels of activated Ras are elevated in human astrocytoma cell lines, secondary to the activation of overexpressed EGF and PDGF receptors, and contribute to tumor growth (A Guha, M Feldkamp, N Lau, G Boss, T Pawson, manuscript submitted for publication). Whether levels of activated Ras are elevated in meningiomas, secondary to signals from growth factor receptors, is not known and needs to be examined in vitro or in tumor specimens by using the enzymatic assay that we recently described (26). It is intriguing to postulate that activation of the overexpressed EGF, PDGF, and other relevant receptors in the tumorigenic meningothelial cells results in activation of Ras, leading to both a mitogenic signal to the nucleus and up-regulation of VEGF/VPF expression, which then lead to neoangiogenesis by paracrine stimulation of endothelial cells. These proposed molecular events, although having a rational basis, need to be tested in experimental paradigms.

The role of VEGF/VPF in meningioma-associated brain edema was also examined, because VEGF/VPF is approximately 10,000 to 50,000 times more potent than histamine in increasing vascular permeability (13, 19). Both the endothelial mitogenic and permeability properties of VEGF/VPF are important for the formation of new blood vessels (17, 19). Increases in vascular permeability are likely mediated through endothelial cytoskeletal rearrangements associated with increased intracellular calcium, allowing opening of endothelial tight junctions, which are crucial for the integrity of the blood-brain barrier in the CNS (10, 13, 17). The vascular permeability induced by VEGF/VPF is postulated to be relevant in the peritumoral edema associated with metastatic tumors to the CNS and GBMs, as well as tumor-related ascitic fluid accumulation (15, 48, 54, 58, 59). Glucocorticoids, the standard treatment for vasogenic tumor-associated CNS edema, are transcriptional inhibitors of VEGF/VPF production (15, 19, 22). Decreasing VEGF/VPF production is one mechanism for the antiedemogenic action of steroids, possibly interrelated with and important to their membrane-stabilizing effects. In this study, the degree of edema was determined from the presenting CT/MRI scans before initiation of steroid therapy, although all specimens were obtained after steroids had been administered for variable lengths of time. The influence of steroids on VEGF/VPF expression in our tumor samples is unknown but must be acknowledged as a potentially significant variable in our results.

In this study, all of the meningiomas associated with marked peritumoral edema (ratio of maximal edema:maximal tumor diameter > 2) had elevated levels of VEGF/VPF expression (*Fig. 5B*). This was best illustrated by our index case, in which the symptomatic meningioma with marked peritumoral edema had extremely high levels of VEGF/VPF, in comparison with the contralateral larger asymptomatic meningioma, of similar histological type, with no peritumoral edema. The lack of VEGF/VPF signal in the asymptomatic meningioma cannot be attributed to its being an autopsy specimen, because a VEGF/VPF signal was easily detected in the autopsy-derived peritumoral brain sample, as demonstrated in *Figure 2*. As an added control, because both of the specimens were from the same patient, the variability of steroid effects on VEGF/VPF expression (as discussed above) is accounted for when these two specimens are compared. In contrast, of the seven meningiomas with minimal peritumoral edema (ratio of maximal edema:maximal tumor diameter > 2), five had low to absent levels of quantified VEGF/VPF mRNA by Northern analysis (*Fig. 5B*). In one of these specimens (from Patient 2), the levels of VEGF/VPF determined immunohistochemically were semiquantitatively graded to be moderately elevated, perhaps reflecting the variability of the sections examined. However, in two specimens (*Fig. 5, LS* and *MP*), little peritumoral edema was evident with elevated levels of VEGF/VPF mRNA and protein (*Fig. 5B*), suggesting that other factors may also be relevant. Obliteration of the subarachnoid plane between the meningioma and brain, with interference with cerebrospinal fluid flow, has been postulated to allow edemogenic tumor factors (such as VEGF/VPF) into the brain parenchyma (30, 43). We therefore postulate that the peritumoral brain edema observed with some meningiomas requires both abundant secretion of VEGF/VPF by the meningothelial cells and a mechanical factor, such as obliteration of the subarachnoid space, that allows for VEGF/VPF to penetrate and act on the peritumoral brain vasculature.